

Investigating Complement Activation in a Cohort of Severely Ill COVID-19 Patients

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Abstract

After its emergence in 2019, COVID-19 has quickly become a leading cause of morbidity and mortality in many nations. Furthermore, the emergence of variants of concern has led to fears that SARS-CoV-2, the virus responsible for COVID-19, could begin evading vaccine acquired immunity. Thus, understanding the factors contributing to severe disease progression is critically important to the public health response and the treatment of severely ill COVID-19 patients. This study examines the role complement protein has in development of severe disease in COVID-19 patients. Complement is a group of proteins that play a critical part of the innate immune system responsible for immune cell recruitment, bacterial lysis, and opsonization of pathogens among many things. To examine the role, complement plays in progression of disease severity in COVID-19 patients, a cohort of hospitalized patients was selected from volunteers admitted to the University of Pittsburgh Medical Center Presbyterian Hospital in Allegheny County, Pennsylvania. Serum from the patients was collected as part of routine medical procedures and excess serum was analyzed via ELISA assays to determine the level of complement factor proteins during disease progression. Following analysis, it was clear that complement factor proteins were significantly elevated in patients with higher body mass index scores. Additionally, it was demonstrated that complement factor proteins were

positively correlated with the ratio of IgG antibodies and IgA antibodies. Furthermore, it was demonstrated that patients with higher ratios of IgG to IgA, and thus higher complement levels, had more severe disease compared to those with higher ratios of IgA to IgG. Together this suggests that IgA is protective against severe disease by down regulating complement activation, and suggesting higher levels of complement activation was a contributor to more severe disease potentially through inflammatory pathways.

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Preface

To begin I would like to thank Dr. Ernesto Marques for providing me with the mentorship and guidance necessary to complete this project during such a unique time. I would also like to thank Dr. Priscila Castanha, who guided me through every procedure and proved to be an invaluable resource when problems arose. Additionally, I would like to thank Jason Yeung, Chen Yang, and Annette Curry who all were phenomenal lab partners not only for their dedication to assisting when necessary but also for making the lab a truly enjoyable place to be. Furthermore, I would like to thank Drs. Douglas Reed and Joshua Matilla for their continued advice, patience, and understanding as members of my essay committee during such a difficult time. Finally, I would like to thank friends, family, and past educators, without whom I would not be the person I am today, I am forever in their debt. The past two years have been filled with grief and suffering due to the SARS-CoV-2 pandemic, and I hope that I can contribute to the ultimate goal of limiting the harm infectious diseases may cause to society.

1.0 Introduction

Beginning in the waning months of 2019 reports began surfacing in Wuhan, Hubei province of a viral pneumonia of unknown etiology caused several people to require mechanical ventilation. By January 2020 the genome of the etiological agent had been sequenced, its association with the disease confirmed by a research team located in China. The viral genome sequence had over 90% genetic similarity to Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV): a respiratory coronavirus which emerged in 2003 and caused over 800 deaths in a 3-month span as it spread rapidly within Southeast Asia and Canada (Xu, Zhao et al. 2020). Cases of the novel coronavirus quickly began to climb within Hubei province in early 2020, prompting the central government of China to implement a complete exclusion zone around Wuhan and mandatory quarantine of known or suspected cases. By March 11 the WHO had declared SARS-CoV-2 a global pandemic, and cases soon began appearing in the United States, beginning in Washington state(2021). To date the SARS-CoV-2 pandemic has infected over 200 million people resulting in more than 4.03 million deaths since its emergence (2021) as of August 12, 2021. With current vaccination rates not expected to result in global herd immunity until 2024 at the earliest, understanding the host response that is leading to severe disease is imperative to create effective preventive measures and treatments to reduce mortality by COVID-19 until sufficient protection is achieved world-wide.

1.1 SARS-CoV-2 and Other Fatal Coronaviruses

Coronaviruses are non-segmented positive sense RNA viruses that are broken into 4 subfamilies with alpha and beta coronaviruses being the 2 genera contribute to the majority of disease in humans. Coronaviruses as a family have been with humans for generations, seldom causing severe disease and thus receiving minimal attention compared to other respiratory pathogens. Prior to the emergence of Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) in southern China's Guangdong province, coronaviruses such as HKU1 and OC43 were mainly responsible for easily manageable 'colds' during winter months (Cui, Li et al. 2019). The 2003 SARS-CoV emergence was particularly alarming to the international community as it was the first deadly coronavirus in modern times, with a calculated case fatality rate (CFR) across all ages of 9.6% as well as the first to efficiently infect the lower respiratory tract leading to acute respiratory distress syndrome (ARDS) in many patients (Zhu, Lian et al. 2020). Additionally, genetic analysis of SARS-like coronaviruses indicated zoonotic transfer from palm civets was likely the cause of initial human infections in the SARS-CoV outbreak of 2003, implicating illegal animal trading and wet markets as industries that could cause future fatal coronaviruses to emerge (Zhu, Lian et al. 2020). For SARS-CoV-2 however, genomic analysis suggests that SARS-CoV-2 emerged from bat populations within China though the exact pathway from bat populations to humans hasn't been fully elucidated (Andersen, Rambaut et al. 2020).

In 2012, 9 years after the emergence of SARS-CoV, a patient in Zarqa, Jordan presented with a similar syndrome that led to the identification of Middle East Respiratory Syndrome coronavirus (MERS-CoV), another beta-coronavirus. Further investigation suggested that MERS,

like SARS-CoV, has a natural reservoir in bats but utilized camels instead of Palm Civets as an intermediary species corroborating the coronavirus's ability to adapt to wide array of fauna to make the jump from bats to humans (Zhu, Lian et al. 2020). Interestingly, SARS-CoV became known for super-spreading events, where one infected individual transmits the virus to dozens of others in a very short period of time. The most famous instance of this occurring in a hotel in Hong Kong where an infected man infected several foreign nationals leading to the virus spreading to several countries (Shen, Ning et al. 2004) This type of super-spreading event also occurred on a flight to Singapore where a single flight attendant infected over 100 passengers leading to a significant number of cases in the country (Al-Tawfiq and Rodriguez-Morales 2020). Luckily, MERS has only had one such well documented event in South Korea (Al-Tawfiq and Rodriguez-Morales 2020) but it has demonstrated an increased mortality rate compared to SARS-CoV with a CFR of 34.3%, although its decreased transmissibility (R_0 of 0.8-1.3) has prevented MERS from spreading rapidly or infecting large population groups the way SARS-CoV did (Zhu, Lian et al. 2020).

Finally, the most recent fatal coronavirus, Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), emerged from central China's Hubei province in the waning months of 2019. Much like SARS-CoV and MERS-CoV, SARS-CoV-2, has well documented cases of superspreading events, a feature that drove infections of both SARS and MERS (Al-Tawfiq and Rodriguez-Morales 2020). However, compared to SARS-CoV and MERS-CoV, SARS-CoV-2 has proven to be less lethal on a per person basis, with a predicted global CFR of 2-3%, though its significantly higher transmissibility has caused exponentially higher casualties compared to MERS and SARS-CoV (Cao, Hiyoshi et al. 2020) . It should be noted that due to the ongoing nature of the

pandemic as well as asymptomatic carriers, the true fatality rate is likely significantly lower than the calculated CFR. Furthermore, the fatality rate is highly variable across nations, as those with higher rates of co-morbidities, or those with decreased healthcare infrastructure have significantly higher fatality rates. Overall, SARS-CoV and SARS-CoV-2 share substantial similarities to one another, compared to SARS-CoV-2 and MERS-CoV. This is exemplified by the genetic similarity of SARS-CoV and SARS-CoV-2 as they share nearly 99% homology between the stalk of the spike glycoprotein in each virus (Xu, Zhao et al. 2020).

In contrast to the other recent coronavirus, the SARS-CoV-2 virus is capable of much more effective transmission. The reasons for the increased transmissibility may be related to the wide spectrum of clinical presentations ranging from mild cough to acute respiratory distress syndrome as well as documented transmission even during asymptomatic and pre-symptomatic stages of disease (Furukawa, Brooks et al. 2020). COVID-19 presents with fever in 77% of cases, cough in 65% of cases, followed by fatigue in 27% and dyspnea in 21% of cases (Sheleme, Bekele et al. 2020). However, in roughly 18% of infected individuals they fail to present with any symptoms (Mizumoto, Kagaya et al. 2020). Overall, the high frequency of asymptomatic cases, the ability to transmit virus during the pre-symptomatic stages and early symptomatic stages, as well as the increased affinity for cellular receptors, are likely to play a significant role in the transmissibility of SARS-CoV-2.

1.2 Current Epidemiology and Intervention Against SARS-CoV-2

SARS-CoV-2 is a respiratory pathogen that is transmitted primarily through airborne and droplet spread from an infected individual. After infection, a patient has an average incubation

period of 6 days, though this can range anywhere from 2 to 21 days, with the infectious period beginning 2 days prior to symptom onset. However, it has also been well documented that SARS-CoV-2 has also been spread effectively by asymptomatic carriers, who may be actively shedding viral particles for up to 21 days (Hu, Song et al. 2020). On average a person is infectious for 8 days post symptom onset, and symptomatic for a similar timeframe. However, severe disease progression follows a much longer course with average hospital admission around day 8 post symptom onset with the most severe cases reaching 28 days or more post symptom onset before disease outcome, tying up hospital resources for a significant amount of time on a per patient basis (Wang, Zheng et al. 2021).

SARS-CoV-2 has been characterized, especially within countries who did not take proactive measures, by exponentially propagating outbreaks, caused principally by populations lacking any meaningful adaptative immune responses capable to protect against SARS-CoV-2. A direct example of the difference proactive measures can make has been demonstrated in the Nordic nations in Northern Europe. Sweden took a laissez-faire approach to the pandemic and as a result, Sweden faced 3 times the cases per capita than the next closest country in the region, Denmark, and had a 5% increase in all-cause mortality over the study period while there was a 5% and 3% decrease in Norway and Denmark during the same period (Yarmol-Matusiak, Cipriano et al. 2021). Recent estimates of R_0 , or the reproductive rate of the virus, place SARS-CoV-2 around 3.6 and 6.1, dependent on interventions imposed by regional authorities as well as emergent variants with some such as the delta variant demonstrating significantly increased transmission (Ke, Romero-Severson et al. 2021). Overall, the original outbreaks followed similar wave patterns of new confirmed cases that have been documented from the 1918 flu pandemic

with similar wave patterns occurring during similar seasonal time frames (He, Zhao et al. 2020). Similar epidemiological curves were present in nations with similar intervention responses and climate, as the United Kingdom's winter wave peaked one day apart from the peak in the United States, further cementing the seasonality of the virus. However, recent emergence of variants of concern have been documented with increased transmissibility by as much as 40%, driving exponential increases in India and the United Kingdom (Davies, Abbott et al. 2021) . The dramatic increase in the United Kingdom is particularly alarming as currently the United Kingdom is one of the most vaccinated countries in the world, yet had increased new daily case counts per capita nearly 400% between May 22 and June 13. Furthermore, a similar spike was noticed in Israel, the most vaccinated western nation, increasing weekly confirmed cases from 221 on the week of June 14 to 15,454 on the week of July 26, a 6992% increase in a little over a months' time. However, it should be noted that deaths in vaccinated regions are significantly depressed compared to unvaccinated regions, and as cases in both countries climb, regions that have high vaccination rates are noticing very little increase in mortality compared to the nearly identical curves between cases and deaths in low vaccinated regions.

Prior to the creation, production and emergency authorization of vaccines, the principal intervention was isolation of infected patients and quarantine of close contacts. This proved effective against SARS-CoV, ultimately leading to its full eradication, however the asymptomatic spread of SARS-CoV-2 as well as lack of public trust and adherence to protocols has limited the effectiveness of these strategies (Wilder-Smith 2020). Furthermore, several countries issued lockdowns and cordon sanitaire, most notably China's initial attempts to keep the virus within the city of Wuhan and the Hubei province, though lockdowns had varying effectiveness among

nations who implemented this strategy depending mostly on the compliance of the population to the lockdown rules. Countries such as China, Japan, and South Korea all instituted strict measures, and were able to decrease their rate of transmission or R_t by 4.78, 3.2, and 4.36 respectively, compared to the United States which instituted mild control measures leading to only a 1.86 reduction in R_t 4 weeks after establishment of community transmission (Gu, Yan et al. 2020). Furthermore, countries who were slow to implement measures had significantly higher average R_t values compared to countries who took quick action at the establishment of community transmission (Gu, Yan et al. 2020). However, nearly every nation instituted mandatory surgical mask requirements for those in public, with this intervention capable of decreasing R_0 below 1.0, the necessary threshold to stop an epidemic, if followed strictly by a large cohort of the population (Howard, Huang et al. 2021). Overall, these interventions, even in countries who were slow to adopt them, have directly saved millions of lives and were capable of controlling the virus within island nations such as New Zealand and Australia.

1.3 Clinical Pathology of SARS-CoV-2

Symptomatic SARS-CoV-2 infection has been summarized via the name Coronavirus Disease 2019 (COVID-19). Severe COVID-19 has disproportionately affected men, especially those with significant comorbidities (Yanez, Weiss et al. 2020). Additionally, older age groups have been impacted by severe disease at significantly higher rates than younger people, with 65+ age group accounting for 46% of hospitalizations and having a 62x higher chance of mortality than those in the 55-64 age group (Yanez, Weiss et al. 2020). Furthermore, the 55-64 age group has

nearly 8x higher mortality rate than those younger than 55, and men in all age groups had 77% higher mortality compared to women (Yanez, Weiss et al. 2020). Moreover, minority populations have faced significantly higher rates of mortality with black and Latino populations having 3.5x and 1.88x the mortality of white counterparts, though this is likely due to societal factors such as minorities making up a larger proportion of essential employees, as well as poor access to healthcare, and mistrust of medical community (Gross, Essien et al. 2020).

The course of the disease tends to follow similar courses to other viral respiratory infections with mild COVID-19 normally begins with a fever, dyspnea, and a persistent cough that is difficult to treat with over-the-counter medication. Additionally, diarrhea, fatigue, and myalgia are also common in mild cases, making it difficult to diagnose from other respiratory infections (He, Cheng et al. 2021). Normally symptoms will resolve within a week, however there have been increasing incidences of 'long-covid', where symptoms, especially fatigue and loss of olfactory senses, will persist for months following viral clearance by the immune system. This is believed to be caused by a number of factors including persistent immune system dysregulation, organ damage from the acute infection, as well as post-intensive care syndrome (Nalbandian, Sehgal et al. 2021). However, the majority of patients will recover in full with limited long-term effects of their acute viral infection.

This is in stark contrast to severe COVID-19 patients who face significant organ damage brought on by cytokine storms, as well as significant mucosal damage to both the upper and lower respiratory tract leading to 'ground glass' lung CT scans and myocarditis in otherwise healthy individuals (Kong, Yang et al. 2020). Significant quantities of complement factors MBL, C3, and C5 among others have been found in immunohistochemistry staining in damaged lung

endothelial cells, as well as in alveolar spaces in damaged lung tissue (Noris, Benigni et al. 2020). Additionally, inhibition of C5a has been shown in mouse models to significantly reduce leukocyte infiltration in tissue samples of MERS and SARS infected mice, suggesting complement pathway activation plays an active role in beta coronavirus lung endothelial pathogenesis (Noris, Benigni et al. 2020). Furthermore, patients have presented with neurological complications including strokes and psychosis brought on by dysregulated inflammation and clotting in the cranium due to acute viral infection. However, fatal COVID-19 progresses into Acute Respiratory Distress Syndrome, necessitating full mechanical ventilation, as well as septicemia and shock leading to multiple organ failure (Shanmugam, Mohammed et al. 2020). Moreover, damaged vasculature associated with deposition of the complement membrane attack complex (MAC), as well as decrease in fibrinolysis, brought on by acute infection, has led to a significant portion of patients developing thrombosis, causing strokes, pulmonary embolisms, and myocardial infarctions in patients months after initial recovery (Price, McCabe et al. 2020). In conclusion it should be noted that clotting, runaway inflammation, cytokine storms, and multiple organ failure have all been linked to dysregulated complement activation, which has been demonstrated in SARS and MERS patients (Noris, Benigni et al. 2020).

1.4 Overview of the Complement System in Response to Viral Infection

Complement is a system of the innate immune response comprised of dozens of soluble proteins that together help to identify, and eliminate pathogens as well as initiate the host inflammatory response (Ricklin, Hajishengallis et al. 2010). In many ways the complement

system is the first line of defense for the host immune response, and its dysregulation or elimination can severely harm the host leading to significant long-term effects.

The complement cascade is activated, as seen in figure 1, by 3 distinct mechanisms that converge at the cleavage of factor 3, leading to the development of the membrane attack complex (MAC). The classical pathway is initiated by bound IgG & IgM immune complexes, leading to the creation of C3 convertase through complement factor 2 and factor 4 cleavage by complement factor 1. On the other hand, the lectin pathway is initiated by mannose binding lectin binding sugars found commonly on pathogen surfaces, initiating cleavage of complement factor 2 and factor 4 through MASP proteins, creating the C3 convertase and activating the cascade. Finally, in the alternate pathway soluble C3b generated from the classical and lectin pathway, binds to complement factor b and cleaved by factor D producing a C3 convertase. The alternative pathway serves mainly to amplify the response generated from the classical and lectin pathways to generate a more robust immune response.

In viral infection specifically, the complement cascade is normally initiated by bound IgG and IgM complexes before complement proteins C3b, C4, and C1q bind the viral surface, inhibiting its ability to bind host cellular structures for cellular entry. Additionally, these complement proteins serve to opsonize a viral particle, facilitating more efficient uptake and destruction by immune cells such as dendritic cells and macrophages (Spear, Hart et al. 2001). However, viruses have proven effective at circumventing complement mediated elimination, with some viruses capable of dysregulating complement activation causing significant inflammation and cellular damage (Stoermer and Morrison 2010).

For viral infection in COVID-19 complement can be both a savior and a menace, leading to effective viral clearance or severe clinical complications. In persons with fatal infections of SARS, another fatal beta-coronavirus, C5a was implicated in alveolar damage leading to fatal organ failure, where reduced levels of C5a were a predictor of potential recovery from ARDS (Wang, Xiao et al. 2015). Furthermore, inhibition of C5aR1 receptor reduced lung injury and myeloid cell derived inflammation in lung tissue suggesting the complement pathway is a critical driver of ARDS in COVID-19 patients (Carvelli, Demaria et al. 2020). Moreover, another common complement activation fragment of all pathways, C3a, is a potent anaphylatoxin known for its ability to cause vasopermeability and leukocyte recruitment in the inflammatory process leading to severe disease pathogenesis when dysregulated. However, it is suggested in murine models that clearance of viral particles is dependent on an effective and efficient complement activation, so it is likely that complete exclusion of the complement cascade would only serve to harm the patient as loss of C3 has been demonstrated to significantly diminish the priming of cytotoxic and helper T-cells in murine models (Dunkelberger and Song 2010). Taken together it is clear that proper functioning complement is crucial to an efficient immune response but dysregulation, specifically increased levels of anaphylatoxins C3a and C5a, can contribute to significant disease progression and cellular damage.

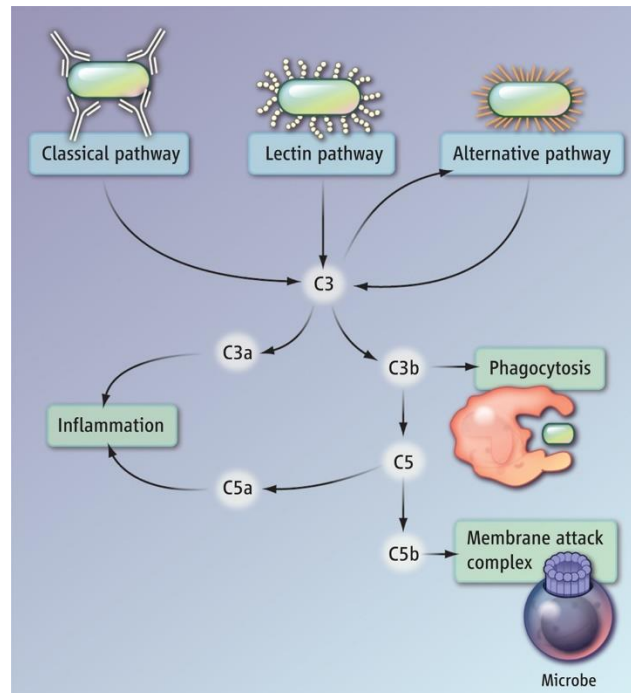


Figure 1 Simplified Overview of the Complement Cascade

This diagram demonstrates the role of the complement system in innate immunity. Activation of any one pathway leads to the activation of C3 and C3 convertase, leading to the deposition of C3a and C3b, facilitating inflammatory responses and phagocytosis respectively. Furthermore, activation eventually leads to the deposition of C5a and C5b, leading to further inflammation and created of the membrane attack complex for cell lysis (Leslie 2012).

2.0 Specific Aims

2.1 Objective 1: Investigate the Relationship Between Complement Activation Levels and Disease Severity in ICU Patients Hospitalized in Allegheny County

Hypothesis: increases in consumption of complement factors 3 and 5 will result in lower levels of these factors in more severe cases while complement activation product, C3a will significantly increase in patients with more severe disease.

Experimental approach: The study is comprised of patients who were PCR confirmed positive that presented at the UPMC emergency department with acute COVID-19 symptoms and were admitted for care. Overall, there are 26 patients comprised of 16 males and 10 females, whose serum was used for all specific aims of the study. Ages range from 41 to 95 and both Caucasian and African-American patients are represented in the data set. Utilizing Enzyme Linked Immunosorbent Assays (ELISAs), the levels of C3, C3a, and C5 levels of the patients will be determined from serum obtained during routine lab tests while hospitalized, with increased levels of all factors suggesting increased complement cascade activation and potentially increased inflammation and immune cell infiltration in endothelial tissue. By measuring C3, C3a and C5 we will be able to determine complement consumption and cascade activation as cleavage of C3 to C3a is an indicator of complement cascade activation, as is the presence of C5. Cross-referencing the determined complement level with known disease severity upon admission will determine if there is a relationship between complement activation levels and disease severity in the admitted patients.

2.2 Objective 2: Investigate how comorbidities affect complement activation in severe COVID-19 patients

Hypothesis: Levels of complement activation will be affected by comorbidities of patients, specifically obesity, cardio-pulmonary conditions and autoimmune disorders. Complement levels are known to be affected by weight as well as chronic conditions, and this hypothesis aims to determine if different complement levels from different chronic conditions effects the level of complement activation and hence the consumption of C3, C5 and elevation of C3a.

Experimental approach: Patient complement levels will be cross-referenced with known comorbidities of patients identified upon admission to determine if there is a relationship between complement activation levels and comorbidities. From the cohort, there were 17 patients with hypertension and 22 with diabetes. Average BMI was 34 for men and 34.59 for women with 20 patients total classified as obese. For analysis, students t-test will be run for each category comparing C3, C3a, and C5 levels between comparison groups, though a higher number of patients may be required to achieve statistical significance($p \leq 0.05$) for some groups. Furthermore, patients were divided by WHO ordinal score with scores above 7 being classified as severe cases, and a score of 10 indicating a fatal outcome. Overall, due to the small size of our cohort population there is limited statistical power of the analysis, though there were still statistically significant results obtained.

2.3 Objective 3: Investigate the Relationship between complement activation levels and Antibody production in COVID-19 severity

Hypothesis: Increased Complement activation will correlate with formation of immune complexes. Complement activation is caused by bound immunoglobulins and high levels of non-neutralizing antibodies can hyperactivate complement leading to more severe disease progression.

Experimental Approach: Utilizing antibody titer data from our group and the data I generated measuring C3, C3a, and C5 levels of severe COVID-19 patients were compared to the humoral response data. Correlation coefficients were calculated to determine any correlative effects between complement activation and antibody titers as IgG immune complexes are a known activator of the complement cascade (Karsten and Köhl 2012). Additionally, groups were split into the same disease severity as objectives 1&2 to determine if any correlation is related to disease severity, either intubation required or not.

3.0 Methods

3.1 Selection of Study Participants

Participants were selected from PCR positive COVID-19 patients at UPMC hospitals in Pittsburgh, Pennsylvania. Study participants agreed to allow leftover serum samples from normal medical procedures to be used in the study. Data on patients was taken by medical professionals within the UPMC system and compiled by CTSI. Study participants were split into 2 groups as seen in table 1, intubated and non-intubated, following the WHO ordinal score scale as seen in figure 2.

Table 1 Demographics of Study Cohort

Demographic table of patients enrolled in the study who are confirmed PCR positive for SARS-CoV-2. Data is broken down by ICU admission status for differing demographic variables relating to the study.

Demographic variable	Non-Intubated patients (WHO score 4-6)	Intubated Patients (WHO score 7-9)	Total
Men	6	10	16
Women	1	9	10
Hypertensive	1	15	16
Diabetes	1	10	11
Obesity	2	18	20
Over 65	5	6	11
Under 65	2	13	15

Patient State	Descriptor	Score
Uninfected	Uninfected; no viral RNA detected	0
Ambulatory mild disease	Asymptomatic; viral RNA detected	1
	Symptomatic; independent	2
	Symptomatic; assistance needed	3
Hospitalised: moderate disease	Hospitalised; no oxygen therapy*	4
	Hospitalised; oxygen by mask or nasal prongs	5
Hospitalised: severe diseases	Hospitalised; oxygen by NIV or high flow	6
	Intubation and mechanical ventilation, $pO_2/FiO_2 \geq 150$ or $SpO_2/FiO_2 \geq 200$	7
	Mechanical ventilation $pO_2/FiO_2 < 150$ ($SpO_2/FiO_2 < 200$) or vasopressors	8
	Mechanical ventilation $pO_2/FiO_2 < 150$ and vasopressors, dialysis, or ECMO	9
Dead	Dead	10

Figure 2 WHO Ordinal Scoring Scale

Mild disease is shaded in green, moderate in yellow, severe in orange, and lethal disease in red (Marshall, Murthy et al. 2020). For our purposes, scores 4-6 will classify the less severe group with 7-9 in the more severe intubated group.

3.2 Complement Protein ELISAs

High Binding, clear, 96 well microplates (Corning) were coated with antibodies in carbonate-bicarbonate buffer in a Styrofoam container at 4° C overnight. Plates were then washed using PBS-T buffer (0.05% tween-twenty in PBS) six times. Plates were then blocked with 150ul of 5% (w/v) bovine serum albumin (Sigma) for one hour at room temperature. The standard curve was then added using 2-fold serially diluted purified complement protein diluted in 1% (w/v)

bovine serum albumin (Sigma) for a total of 10 dilutions. Normal human serum (Complement Technology) and a blank of 1% (w/v) bovine serum albumin was added before the plate was incubated for 2 hours at room temperature. The plate was then washed 6 times with 1X PBS. Corresponding detection antibody was then added diluted in 1% (w/v) bovine serum albumin (Sigma) and left to incubate for 1 hour at room temperature. The plate was then washed 6 times with 1X PBS. Horseradish peroxidase diluted in 1% (w/v) bovine serum albumin (Sigma) was then added to the plate and left to incubate at room temperature for 1 hour. The plate was then washed 6 times with 1X PBS before 50 ul of room temperature TMB was added to each well for 20 minutes at room temperature. After the 20 minutes expired 1M hydrochloric acid (HCl) was added to each well. The plate was then read at 450 nm using a SpectraMax Plus PC380 microplate spectrometer using SoftMax Pro Software (Molecular Devices). Concentrations were calculated using logarithmic regression from GraphPad Prism statistical software.

3.3 Patient Serum Complement ELISA

High binding, clear, 96 well plates (Corning) were coated with C3, C3a, or C5 capture antibodies diluted in carbonate bi-carbonate buffer and left covered at 4°C overnight. After being left overnight, plates were washed 6 times with 0.05% by volume tween twenty in Phosphate buffer saline (PBS) wash solution before being blocked with 5% (w/v) bovine serum albumin (BSA; Sigma) diluted in PBS and left for 1 hour at room temperature. Subsequently plates were washed with wash solution before patient serum samples 2 times serially diluted in

1% (w/v) BSA were added along with standard curves and positive and negative controls. Plates were then left for 2 hours at room temperature before being washed 6 times with wash solution. Corresponding detection antibody diluted in 1% BSA (w/v) was then added to the plate before being left 1 hour at room temperature. Following this incubation, the plate was washed 6 times with wash solution before horse-radish peroxidase diluted in 1% BSA(w/v) was added and left for 1 hour at room temperature. This was followed by another 6 times wash with wash solution before room temperature TMB was added and left for 20 minutes. This reaction was stopped after 20 minutes with 1M Hydrochloric acid, and the plate was read at 450 nm using a SpectraMax Plus PC380 microplate spectrometer using SoftMax Pro Software (Molecular Devices). Concentrations were calculated using logarithmic regression from GraphPad Prism statistical software.

4.0 Results

4.1 Objective One: Establishing a Relationship between Complement Activation and Disease Severity within ICU Patients

4.1.1 Determining the effect of Complement Activation on Disease Severity on Admission to ICU and Disease Severity Peak During Hospitalization

ELISAs were performed on the cohort to determine levels of C3, C3a, and C5 upon admission to the hospital and how this correlated with disease severity. All patient serum showed conversion of C3 to C3a as well as consumption of C5, which is to be expected of a patient with an acute viral infection. These concentrations were analyzed compared to a patient's World Health Organization Ordinal Score, which can be seen in figure 2, to ensure all patients were scored with minimal bias when determining disease severity. As seen in Figures 3 A and C, none of the complement factor proteins significant correlated with disease severity upon admission to the hospital. However, in other studies within our group there has been correlation between complement consumption levels and disease severity when the full scope of disease severity is taken into account and not just severely ill patients. Overall, C3 complement activation was not significantly ($p=0.8231$) correlated with disease severity upon admission. Furthermore, C3a concentration levels were not significantly correlated with disease severity ($p=0.2293$). Finally, C5 concentrations were not significant between disease severity groups ($p=0.9008$). It should be noted however, that due to the small sample size and narrow focus of disease severity there was limited statistical power in the data. Furthermore, this data specifically looks at score upon admission, which is not indicative of the full disease progression as many patients had worsening disease severity post-admission.

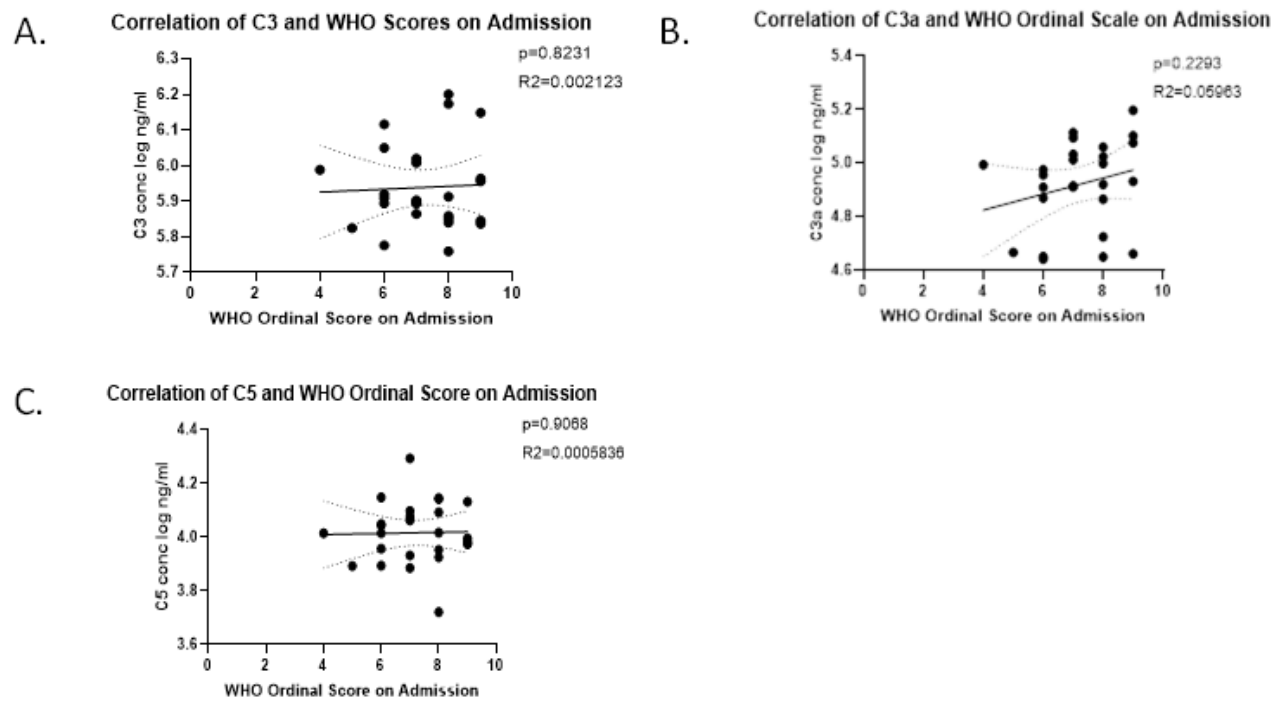


Figure 3 Correlation of Complement Factor Levels and Ordinal Score on Admission

WHO ordinal scale used as a standard weighting of disease severity. A) C3 complement levels, no significant correlation seen. B) C3a complement levels, weak but not significant correlation between disease score and C3a levels. C) C5 complement levels, no correlation seen between disease severity and complement levels.

4.1.2 Determining the Relationship Between Complement Activation and Progression to more Severe Disease

Due to complement being a critical initiator of pathways leading to adaptive immunity, and thus antibodies, it is possible that complement concentrations could effect the overall disease progression of patients. As seen in figure 4, the ratio of C3 and C3a weakly negatively correlates with the worst disease score of the patients during hospitalization while C3 and C5 alone don't

correlate either way. Overall, consumption of C3 to C3a, and subsequent presence of C5, correlated with highest obtained disease severity, though not significantly. It should be noted however, that this data only examines a narrow spectrum of disease and investigation into differences in mild and asymptomatic disease should be explored.

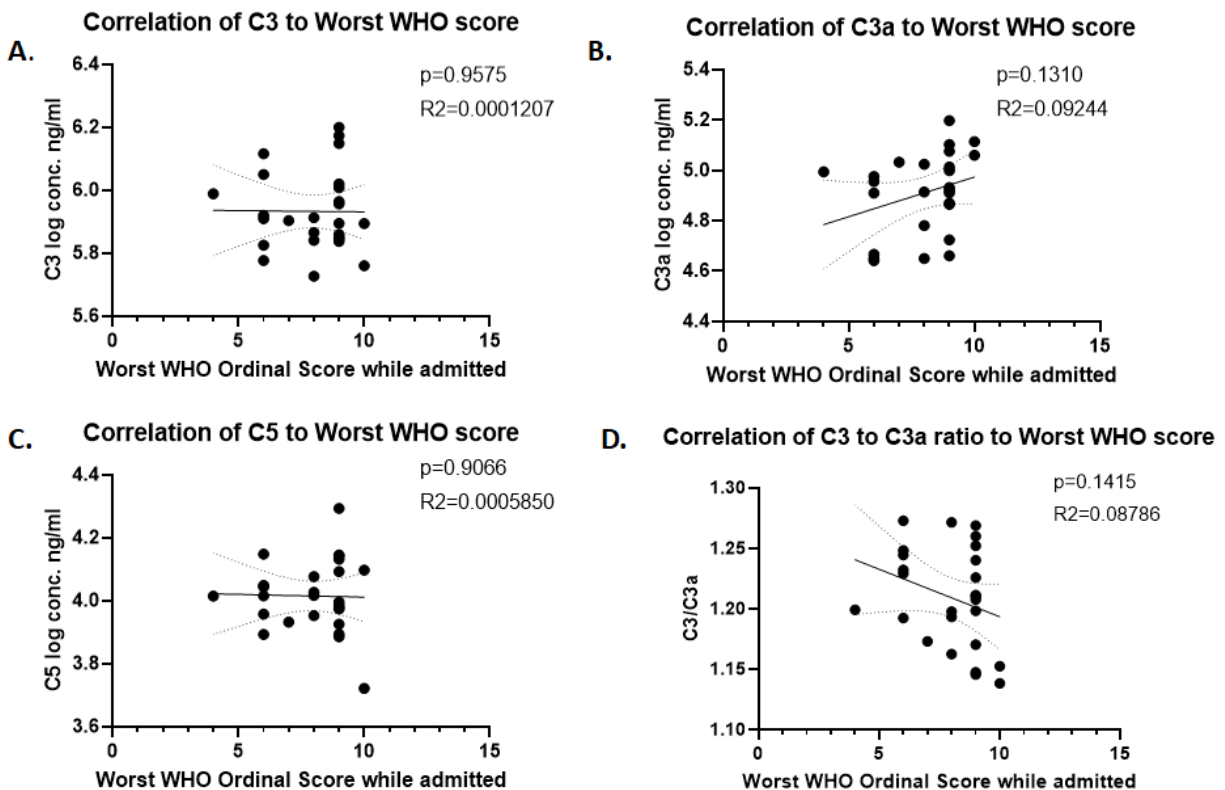


Figure 4 Correlation of Complement Factor Levels and Worst Ordinal Score During Hospitalization

ELISAs of patient sera were performed to gauge concentration levels. A) No correlation between C3 concentrations and WHO scores. B) Weak positive correlation between C3a and WHO scores but not statistically significant likely due to small sample size C) No correlation between C5 levels and WHO scores. D) Weak correlation between the ratio of C3 to C3a vs WHO scores but not statistically significant likely due to small sample size.

4.2 Objective 2: Determining the Effects of Comorbidities on Complement Activation in Severe COVID-19 Patients

4.2.1 Investigating the effect of Non-cardio Pulmonary Comorbidities on Complement Activation

It has been well described that complement levels, and complement activation can be affected by comorbidities common in the American population, such as obesity. C3 and C5 have been shown to have increased concentrations in obese patients, potentially leading to higher levels of inflammatory disease (Pomeroy, Mitchell et al. 1997). This is seen in figure 5a&b, as C3 and C3a moderately correlates with obesity levels, while in figure 5c C5 has weak non-statistically significant correlations. This data demonstrates that obesity is correlated with higher levels of C3 and C3a among the cohort of hospitalized patients.

Obesity also is linked to many chronic conditions, including diabetes, where the increased levels of pro-inflammatory cytokines, glycerol, and NEFA significantly increase insulin resistance in patients leading to the development of type II diabetes (Al-Goblan, Al-Alfi et al. 2014).

Development of diabetes can then lead to dysregulated glucose levels in the body, with unregulated insulin levels causing spikes in blood glucose (Al-Goblan, Al-Alfi et al. 2014).

Furthermore, high levels of glucose can inhibit C3 deposition on pathogen surfaces, suggesting unregulated diabetes could inhibit complement conversion from C3 to C3a (Hair, Echague et al. 2012). As seen in figure 6 however, there was not a significant difference between complement factors of diabetic or non-diabetic patients, although for C3, C3a and C5, mean complement factor levels are higher in the non-diabetic group. Taken together the data from specific aim 2 suggests that BMI is the main effector on complement activation levels with less severe patients having lower BMI and thus lower levels of complement factor 3.

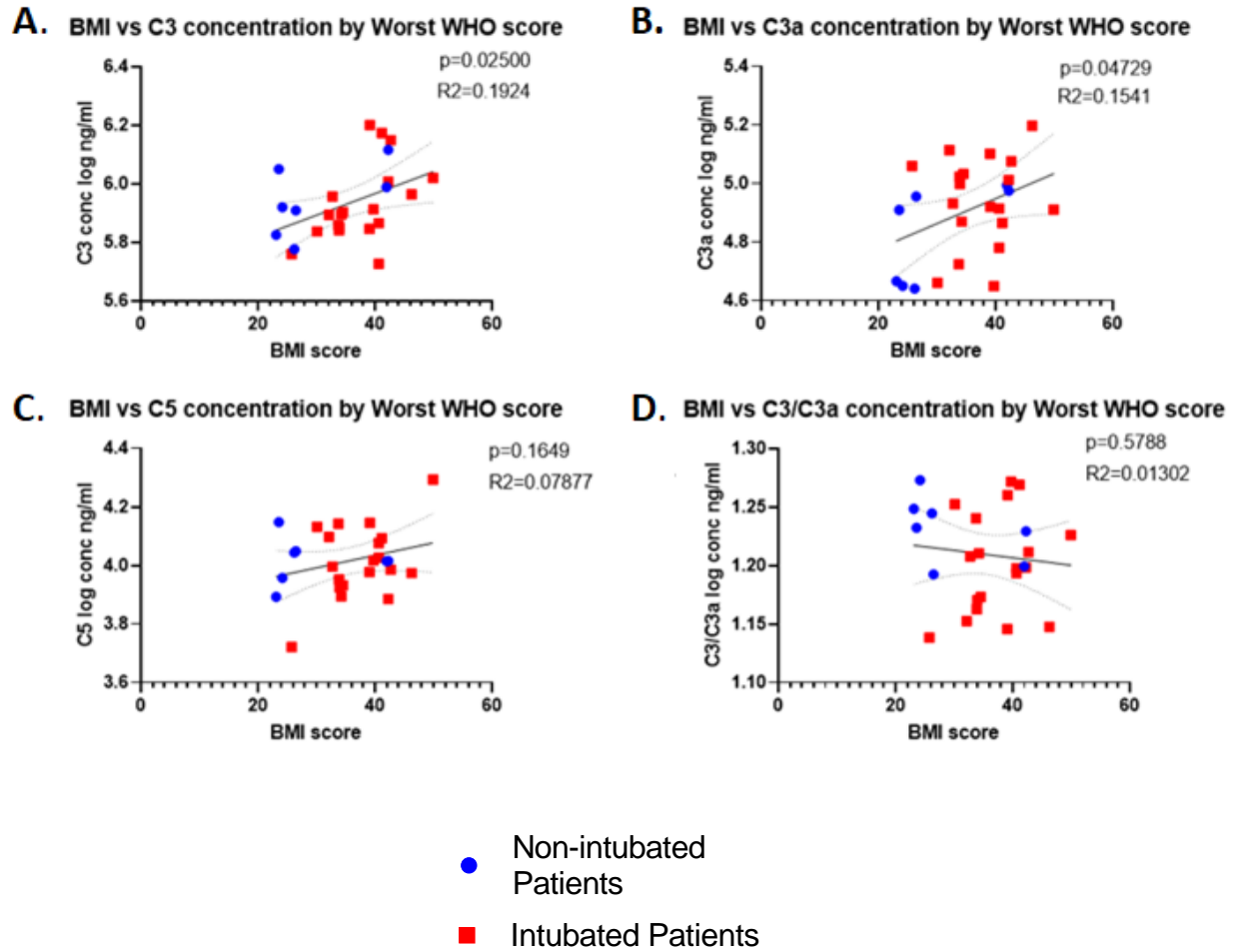


Figure 5 Correlation of Complement Factors and BMI score by Disease Severity

BMI is significantly correlated with C3 and C3a concentrations (Fig A&B). C) Weak positive correlation between complement and C5 concentrations with no statistical significance. D) No correlation between C3/C3a ratio and BMI. For all figures patients who were not intubated clustered towards the lower BMI levels.

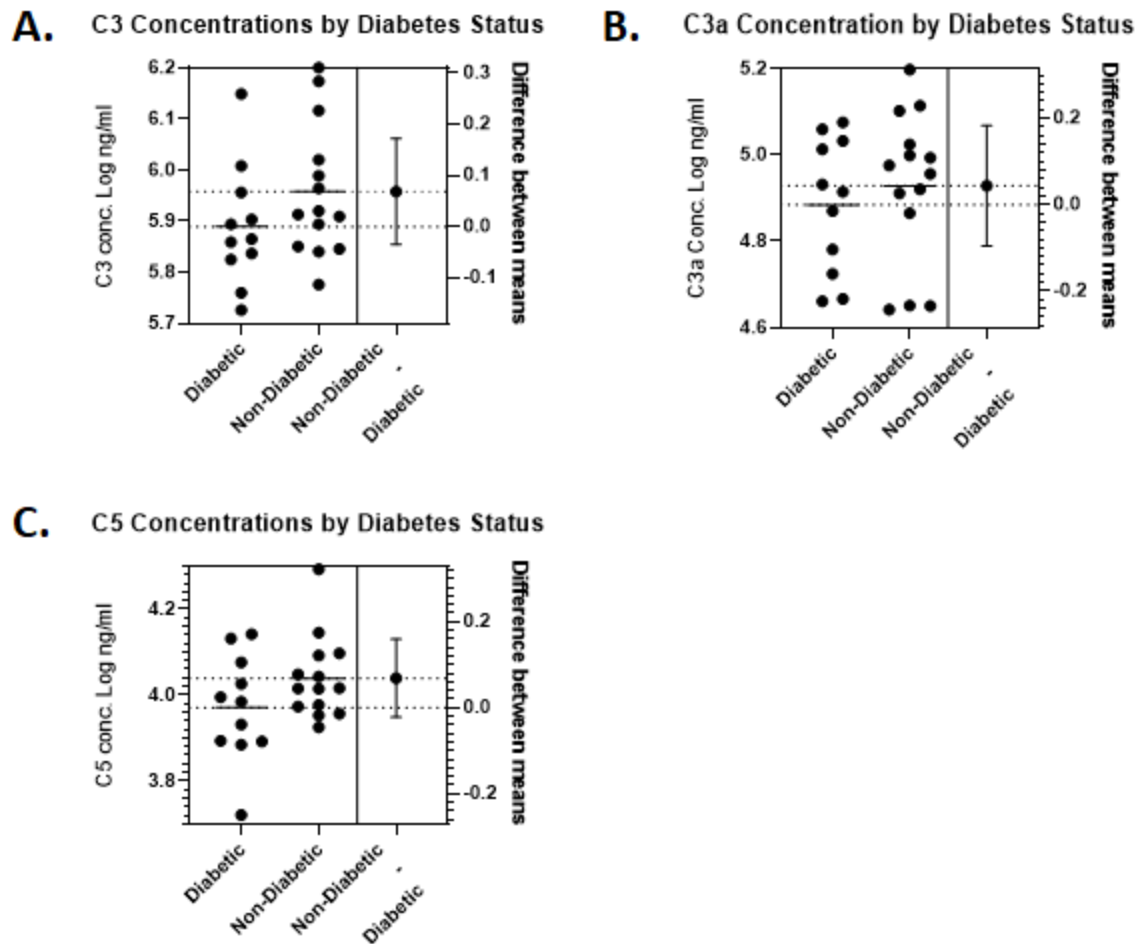


Figure 6 Complement Factor Levels by Diabetic Status

A) Slight increase in C3 concentrations in those with diabetes though no statistically significant difference. B) Slight increase in C3a concentrations in those with diabetes though there is no statistically significant difference. C) slight increase in C5 concentrations in those with diabetes though there is no statistically significant difference.

4.2.2 Complement Activation in relation to cardio-pulmonary comorbidities in severe COVID-19 patients

Hypertension and heart disease increase risk of severe disease in COVID-19 patients with an adjusted odds ratio of 2.36 for patients developing severe disease with cardiopulmonary disease (Yang, Zheng et al. 2020). As seen in figure 7, our data supports that patients with

hypertension are at a higher risk of severe disease, as hypertensive patients cluster towards more severe WHO Ordinal Scores upon admission. However, when investigated with complement there is no significant difference in complement levels among hypertensive patients and non-hypertensive patients though the small sample size could negatively affect statistical significance. Furthermore, there were limited differences in COPD complement concentrations in the patient cohort.

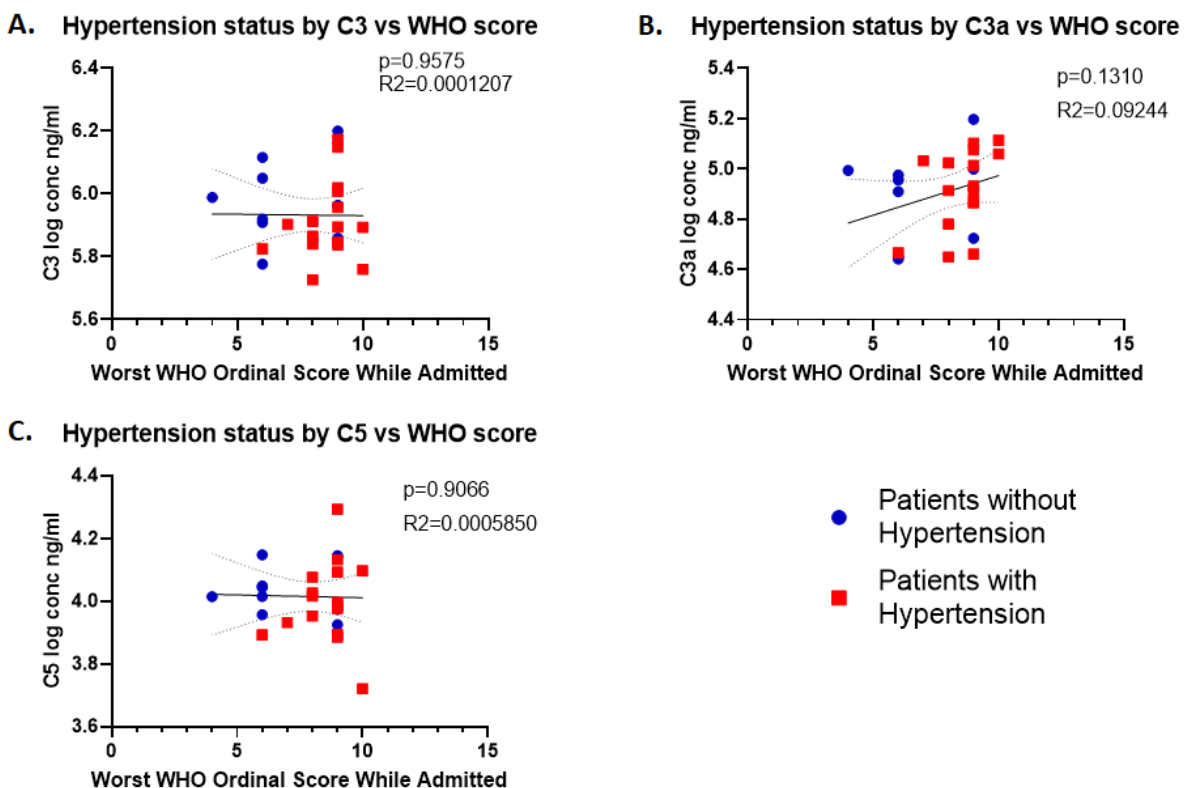


Figure 7 Correlation of Complement Factor Levels and Worst Ordinal Score While Hospitalized by Hypertensive Status

A) There is no correlation between C3 and WHO score on admission. B) there is very weak correlation between C3a levels and WHO score on admission but it is not statistically significant C) there is no correlation between C5 and

WHO score on admission. For all figures patients without hypertension cluster towards the lower WHO score on admission.

4.3 Objective 3: Investigating the Relationship Between Complement Activation and the Adaptive Immune Response in Severe COVID-19 Patients

4.3.1 Conversion ratios and the Adaptive Immune Response

Complement can serve as an initiator of immune response and it has been documented that effective complement conversion is necessary for development of humoral immunity against pathogens (Dunkelberger and Song 2010). Furthermore, IgG immunity can create a feedback loop increasing complement activation and inflammation, while IgA is also capable of activating the complement cascade through Mannose-binding lectin (Rizk, Maillard et al. 2019). This is supported by the data shown in COVID-19 patients in figure 8, as higher ratios of IgG led to increased complement activation demonstrated by the lower levels of C3 compared to higher levels of C3a in the cohort as seen in figure 8, with less severe patients having higher IgA ratios and thus decreased C3 to C3a conversion compared to patients with immune responses producing higher ratios of IgG though the low number of patients is likely responsible for the lack of statistical significance.

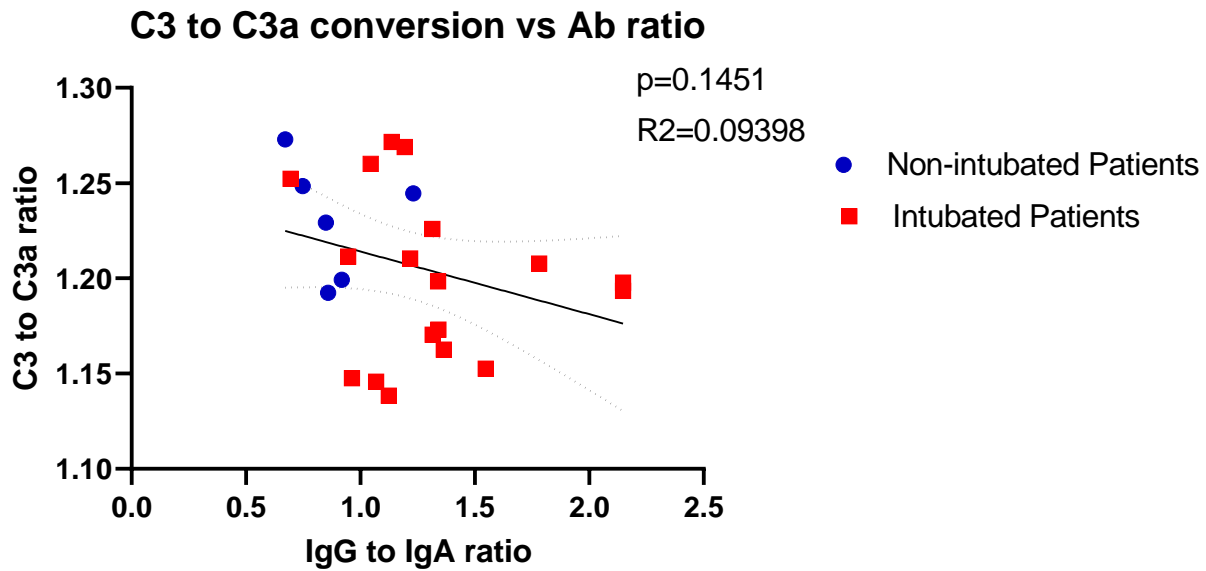


Figure 8 Correlation of Complement Activation to Antibody Ratio by Disease Severity

More severe patients cluster towards higher ratios of IgG to IgA and higher rates of C3 to C3a conversion indicated by a lower C3 to C3a ratio. The correlative data is not significant however.

4.3.2 Effects of Complement and Antibody Concentrations on Disease Severity in Severe COVID-19 Patients

Finally, the effects of complement, and antibody concentrations were analyzed for the effects on disease severity. Antibody titers were also investigated to determine if there was a relationship between complement factor concentrations and effective viral binding and neutralization from humoral immunity. As seen in figure 9a, there is a moderate, statistically significant, positive correlation between serum IgA concentrations and factor 3 levels. Furthermore, as seen in Figure 9b, IgA titers are weakly negatively correlated with C3a levels suggesting that higher IgA concentrations may be tied to C3 conversion to C3a in COVID-19 patients, though this data was not statistically significant. Taken together this suggests that IgA

potentially inhibits consumption of C3 and production of C3a, and through complement down regulation potentially protects from more severe disease. However, as shown in Figure 10 there is no significant correlation between complement C3, C5 and C3a with neutralization titers suggesting viral neutralization is not highly effected by complement in severe COVID-19 patients. Finally, in Figure 11a , it is shown that anti-spike glycoprotein IgG significantly correlates with neutralization of SARS-CoV-2 virus comparative to both anti-nucleocapsid antibodies as well as anti-spike glycoprotein IgA, suggesting that the potentially protective effects seen in Figure 9 from IgA is likely not a result of IgA neutralizing SARS-CoV-2 but preventing excessive complement activation and inflammation. Overall, the data obtained suggests that IgA potentially down regulates complement activation, and IgA is not responsible for viral neutralization, indicating any therapeutic effects are potentially a results of complement down-regulation.

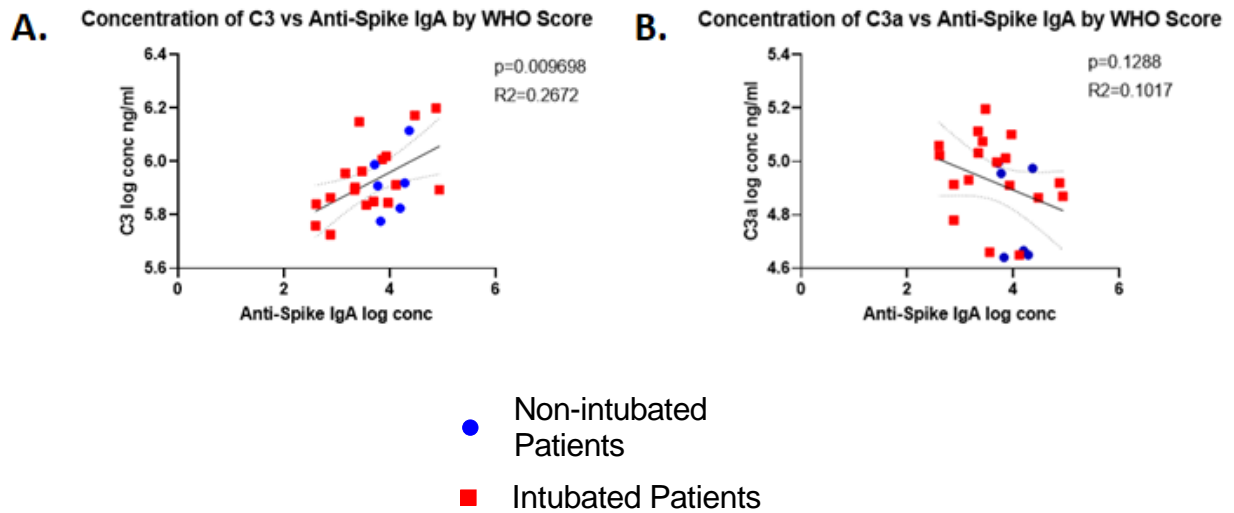


Figure 9 Correlation of Complement Factors to IgA concentrations by Disease Severity

Anti-Spike glycoprotein IgA demonstrated to have immuomodulating effects on complement cascade activation and conversion of C3 to C3a. A) C3 concentrations are moderately correlated with IgA concentrations. Additionally, less severe patients seem to cluster towards higher IgA levels. B) There is a weak negative correlation between C3a and IgA though the relation is not statistically significant.

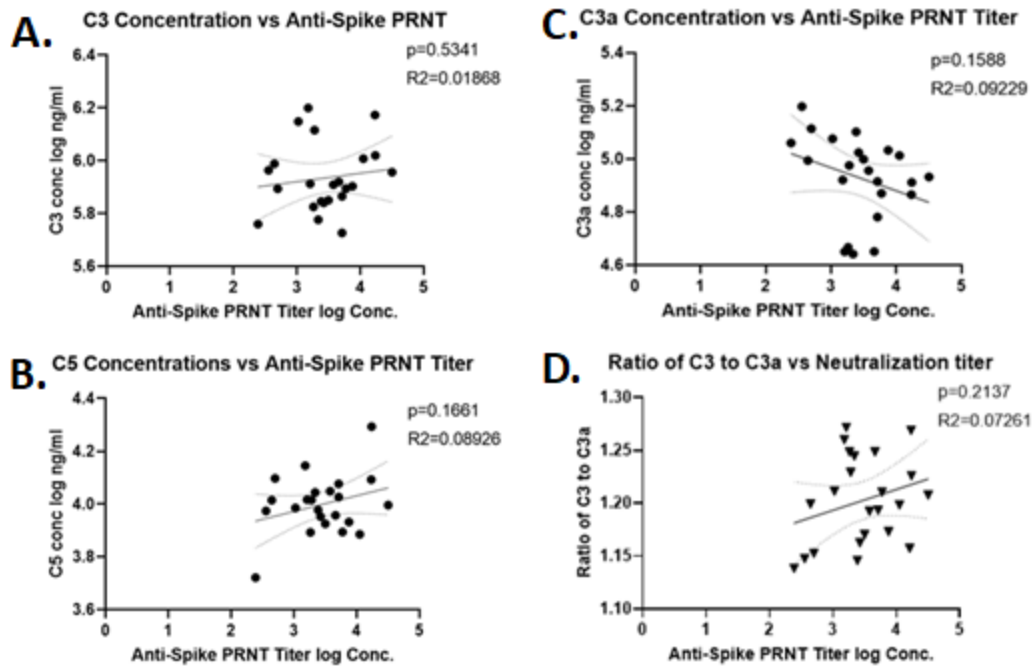


Figure 10 Correlation of Complement Factors to PRNT Titers

Complement concentrations in relation to in vitro plaque reduction neutralization test titers are non significant, suggesting that complement concentration is slightly correlated with viral neutralization. A, B, C, D) None of the complement proteins investigated have a significant correlation with PRNT titers.

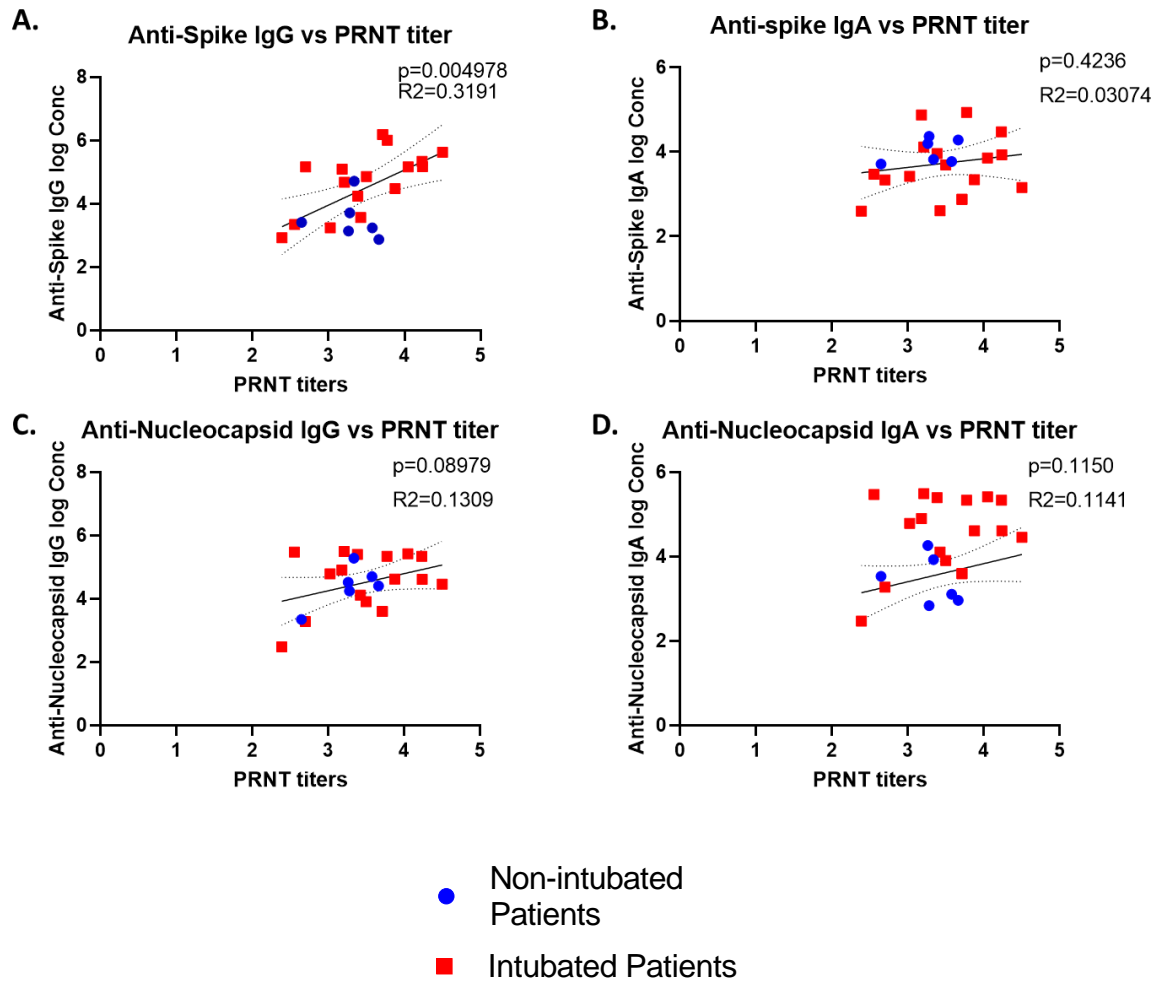


Figure 11 Correlation of Antibodies to PRNT Titers by Disease Severity

A) There is a moderately positive correlation between anti-spike IgG and neutralization titers indicating that IgG is a driver of viral neutralization. B, C, D) there is no significant correlation among Anti-Spike IgA or Anti-nucleocapsid IgG and IgA and viral neutralization titers.

5.0 Discussion: The Findings and Implications of Complement Activation in Severe COVID-19 Patients

Overall, it has been demonstrated in the literature that complement activation has been a significant contributor to disease severity, primarily through recruitment of immune cells, and thus inflammatory molecules, into endothelial tissue (Java, Apicelli et al. 2020). Additionally, retroactive studies have demonstrated that obesity is a significant risk factor for severe disease progression with an adjusted risk ratio of 1.42 compared to healthy patients (Poly, Islam et al. 2021). These factors taken with the data in figure 5a and 5b suggests that it is possible that increased complement activation associated with obesity is a contributor to worse disease progression among obese patients as seen in meta-analyses of COVID-19 patients.

Apart from comorbidities, the adaptive immune response plays a critical role in regulation and activation of the complement cascade during viral infection. It has been well documented throughout the literature that bound immune complexes, specifically IgG and IgM activate the complement cascade through the classical complement pathway (Diebolder, Beurskens et al. 2014). In contrast it has also been documented that bound IgA can inhibit activation of the classical complement pathway as a regulatory mechanism and thus reduce inflammation during viral infection (Russell, Reinholdt et al. 1989). The interplay between IgG activation and IgA regulation of complement activation in COVID-19 patients was weakly suggested in figure 8 as increased IgG serum level were weakly associated with increased activation though this data was not significant. However, when looking specifically at the regulatory effects of IgA there is significant positive correlation between serum IgA and factor 3 levels suggesting that the

regulatory function of bound serum IgA is potentially reducing complement activation in COVID-19 patients and thus potentially reducing the deleterious inflammatory response.

Finally, it has been documented that serum IgG and IgA significantly correlates with viral neutralization in both severe and non-severe COVID-19 patients (Chen, Tong et al. 2020). In figure 11 it is demonstrated that serum IgG is associated with viral neutralization in our cohort of patients as well. Additionally, it is seen in figure 11 that IgA does not correlate with viral neutralization, possibly due to the limited scope of disease investigated in the study or the small number of study participants, though it is expected that serum IgA would contribute to viral neutralization.

Together the data from this study suggests that there is a complex interplay between comorbidities and humoral immunity when it comes to complement activation in COVID-19 patients. It is possible that conditions such as obesity have worse disease progressions due to their increased complement activation states, thus leading to greater inflammation and cellular recruitment in endothelial tissues. Furthermore, our data suggests that IgA is inhibiting complement activation in COVID-19 patients, potentially modulating severe inflammation caused by complement overactivation though further tissue studies should be pursued to gauge the true *in vivo* effects. Finally, the data from the PRNT assays suggested that serum IgA affected disease progression through means other than viral neutralization, though it is likely some neutralization by serum IgA occurs.

Overall, the study faced limitations in the conclusions that were able to be drawn, specifically from the low number of participants and the narrow scope of disease severity investigated. Moving forward, tissue studies into lung endothelial infiltration should be done to

determine if increased levels of serum IgA modulate infiltration of immune cells as well as cellular complement deposition. Furthermore, widening the scope of disease studied may provide increased insight, and statistical power, to correlative relationships investigated in this study. Finally, the effects of new variants, principally the delta variant, should be investigated in relation to complement activation to determine how they may alter complement activation and disease progression.

6.0 Significance to Public Health

COVID-19 has continued to strain public health infrastructure on every inhabited region. Recent emergence of variants demonstrating increased ability to cause severe disease among younger groups has caused rapid increases in hospitalizations in recent weeks in even highly vaccinated regions. Furthermore, vaccine hesitancy has allowed for the emergence of new variants as well as unmitigated spread of SARS-CoV-2 throughout communities. Throughout this, it has become apparent that the ability of medical professionals to treat acute SARS-CoV-2 infection is important to reduce mortality associated with acute infection. By understanding mechanisms that contribute to significant disease progression, it can be possible to develop novel therapeutic approaches to decrease disease severity among the hospitalized portion of the population. Finally, should there be an emergence of another pandemic beta-coronavirus in the future, elucidation of disease causing pathways of beta-coronaviruses could significantly improve the collective public health response in treating severe disease before prophylactic measures are available. Further study of complements role should get a larger sample size in more regions of the US to reduce error or bias in the study.

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